Transport of Exogenous Growth Factors and Cytokines to the Cytosol and to the Nucleus

SJUR OLSNES, OLAV KLINGENBERG, AND ANTONI WIEĐŁOCHA

Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo, Norway

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Olsnes, Sjur, Olav Klingenberg, and Antoni Wieđłocha. Transport of Exogenous Growth Factors and Cytokines to the Cytosol and to the Nucleus. Physiol Rev 83: 163–182, 2003; 10.1152/physrev.00021.2002.—In recent years a number of growth factors, cytokines, protein hormones, and other proteins have been found in the nucleus after having been added externally to cells. This review evaluates the evidence that translocation takes place and discusses possible mechanisms. As a demonstration of the principle that extracellular proteins can penetrate cellular membranes and reach the cytosol, a brief overview of the penetration mechanism of protein toxins with intracellular sites of action is given. Then problems and pitfalls in attempts to demonstrate the presence of proteins in the cytosol and in the nucleus as opposed to intracellular vesicular compartments are discussed, and some new approaches to study this are described. A detailed overview of the evidence for translocation of fibroblast growth factor, HIV-Tat, interferon-γ, and other proteins where there is evidence for intracellular action is given, and translocation mechanisms are discussed. It is concluded that although there are many pitfalls, the bulk of the experiments indicate that certain proteins are indeed able to enter the cytosol and nucleus. Possible roles of the internalized proteins are discussed.

I. INTRODUCTION

Protein hormones, growth factors, and cytokines bind to receptors at the cell surface and act by transmitting a signal to the interior of the cell. The receptors are most often transmembrane proteins, and the signaling often implies the activation of an enzymatic activity carried by the intracellular domain of the receptor protein as such, or by a protein that is associated with the receptor or that becomes associated with it upon binding of the ligand to the extracellular part of the receptor. These processes have been worked out in great detail and are not reviewed here. It is generally assumed that the protein hormones and growth factors stay at the cell exterior or that they are taken up by endocytosis and may continue to signal for a while until they are eventually degraded (192). In both cases it is assumed that the hormones, cytokines, and growth factors are separated from the cytosol by a membrane. Steroid hormones, thyroid hormones, retinoic acid, and other lipophilic compounds are able to penetrate cellular membranes and act by binding to proteins in the cytosol or in the nucleus, resulting in gene regulation. The two classes of hormones and growth factors are considered as fundamentally different in this respect.
Over the years it was reported repeatedly that also protein hormones, cytokines, and growth factors were found intracellularly, usually in the nucleus. Many of these claims were based on data obtained with immunofluorescence or cell fractionation, approaches that can easily give misleading results, as discussed below. However, a number of studies where more sophisticated techniques were employed have provided evidence that at least some of these proteins are indeed able to penetrate into cells. Some evidence for intracellular action of the growth factors has also appeared. Although it is still not generally accepted that protein hormones and growth factors may act intracellularly, sufficient data have accumulated to justify a critical review of the existing literature.

Transport of proteins across cellular membranes has proven to be a complicated process in all cases where the mechanism has been worked out in detail. Transport of export proteins into the lumen of the endoplasmic reticulum (85, 133, 171) and of mitochondrial proteins into mitochondria (98, 214) requires complicated translocation machines involving a number of protein components. In both cases the proteins must unfold to some extent before translocation can take place. Folded proteins may be able to translocate into chloroplasts (76) and peroxisomes (33), but also here complicated machineries are involved. The idea that proteins should be able to translocate through the plasma membrane or across the membranes of intracellular membrane-bound organelles therefore seems unlikely unless a translocation apparatus is available.

A number of short basic peptides have been reported to induce translocation of proteins across membranes. These peptides, which originate from antennapedia (41, 165–167), from HIV-Tat transactivating protein (147), and from other sources were chemically linked or fused by recombinant technology to various proteins with known intracellular activities. When added to cells, they induced biological effects consistent with their known function. The procedure has been tested out in a large number of settings with positive results. So far quantitative assessments of the amount of translocated protein has not been reported, and the mechanism of entry remains largely unknown.

Recently, it has become clear that misfolded proteins in the endoplasmic reticulum are not exported from the cell, but retro-translocated into the cytosol where they are degraded by proteasomes (234, 236). Several components used for export of proteins into the endoplasmic reticulum, such as the sec61 complex, are involved in this reverse translocation.

II. TRANSLLOCATION MECHANISMS USED BY PROTEIN TOXINS

Before discussing translocation of growth factors and cytokines, we briefly review how certain protein toxins from poisonous plants and from pathogenic bacteria penetrate into the cytosol. It is possible that related mechanisms can be employed in the translocation of physiological proteins. Some of these toxins employ cellular mechanisms for translocation, whereas others carry their own translocation apparatus. In neither case is the translocation a simple process.

A. Toxins That Carry Their Own Translocation Apparatus

Diphtheria toxin is the pathogenicity factor in diphtheria (36, 155). The active form of the toxin consists of two disulfide-linked polypeptides with different functions. The one polypeptide, the A-fragment, is the toxic component. It is an enzyme that enters the cytosol and inactivates elongation factor 2 required for protein synthesis. This is achieved by adding an ADP-ribose residue onto a unique amino acid, diaphamide, found in the elongation factor. As a result, protein synthesis is blocked and the cell eventually dies.

The A-fragment is by itself essentially nontoxic. Only when given to cells in concentrations more than 1 06 times higher than those required for whole toxin is some toxic effect observed.

The B-fragment consists of two functionally different domains. The R-domain, which is located at the COOH terminus, binds the toxin to cell surface receptors that are uncleaved precursors of heparin-binding epidermal growth factor (EGF)-like growth factor (148). The remaining part of the B-fragment, the T-domain, is almost entirely α-helical (29) and inserts into membranes upon triggering by low pH (183). The toxin meets low pH in endosomes after the surface-bound toxin is taken up by endocytosis.

The translocation process is initiated by the insertion of two of the α-helices (8 and 9) of the T-domain into the membrane (139, 190). At the tip of the loop separating these helices there are two acidic amino acids that must be protonated before the helices can insert into the membrane. This protonation normally occurs in the acidic endosomes, but it can also occur at the cell surface if the cells are exposed to medium with low pH (182). The insertion of the helices into the membrane induces a process that eventually pulls the A-fragment across the membrane. A rather extensive unfolding of the A-fragment is required for the translocation to occur.

The toxin is also able to translocate passenger proteins. Thus acidic fibroblast growth factor (aFGF) (233) and dihydrofolate reductase (93) fused to the NH2 terminus of the toxin A-fragment were efficiently translocated into the cytosol by the toxin pathway. Even the passenger protein must unfold for translocation to occur. The inability of many proteins to unfold at the low pH of the
endosomes is probably the reason why only few proteins are able to be translocated by the toxin.

Another toxin that carries its own translocation apparatus, anthrax toxin, has a completely different structure and mechanism of action. This toxin is synthesized by *Bacillus anthracis* and consists of three protein entities (107). The protein called the protective antigen (because antibodies against it protect against the toxin) is proteolytically cleaved to yield a polypeptide that aggregates to form a heptameric ring structure that binds to cell surface receptors (21) and forms binding sites for the two other components termed edema factor and lethal factor (107). These two proteins compete for the same binding site on the heptameric ring structure. After the binding has taken place, the complex is endocytosed and, like in the case of diphtheria toxin, the low pH in the endosomes induces translocation of the toxic entities, the edema factor and the lethal factor, into the cytosol (225). The edema factor is an adenylate cyclase that is activated by calmodulin (108), whereas the lethal factor is a metalloprotease that cleaves the mitogen-activated protein kinase (MEK) (44, 217). Both actions interfere with the ability of the macrophages to kill the anthrax bacilli, which are therefore able to grow uninhibited in the blood with production of large amounts of toxin with often fatal results.

Also, anthrax toxin is able to translocate passenger proteins to the cytosol, and also in this case, extensive unfolding of the proteins is a requirement for translocation (225).

B. Toxins Employing Cellular Mechanisms for Entry

A number of toxins are not equipped with their own translocation apparatus, and still they are able to translocate an enzymatically active subunit to the cytosol. To this group belong the plant toxins abrin and ricin and the bacterial toxins cholera toxin and Shiga toxin. Also these toxins bind to receptors at the cell surface by their B-moiety (21) and forms binding sites for the two other components termed edema factor and lethal factor (107). In the case of cholera toxin and Shiga toxin, this complex is endocytosed and transported retrograde to the Golgi apparatus (215) and from there to the endoplasmic reticulum (225). The edema factor is an adenylate cyclase that is activated by calmodulin (108), whereas the lethal factor is a metalloprotease that cleaves the mitogen-activated protein kinase (MEK) (44, 217). Both actions interfere with the ability of the macrophages to kill the anthrax bacilli, which are therefore able to grow uninhibited in the blood with production of large amounts of toxin with often fatal results.

In the case of abrin, ricin, and Shiga toxin, this consists of removing an adenosine residue from the 28S RNA of the large ribosomal subunit (48–50). Although this does not result in disruption of the RNA backbone, elongation factors are unable to bind properly to the modified ribosomes and protein synthesis stops.

The A-chain of cholera toxin is an ADP-ribosylating enzyme that modifies G proteins and thereby increases the level of cAMP in the cells (61).

In addition to the toxins here mentioned, there are a large number of protein toxins that enter the cytosol (7). In some cases this occurs in similar ways as here described, but in most cases the mechanism has not yet been elucidated. The translocation appears, however, to be a complicated process in all cases.

Because a large number of bacteria and plants have been able to form protein toxins that are able to translocate an enzymatically active protein efficiently to the cytosol using different approaches, this cannot be a too difficult task. Therefore, it would be a priori not be unlikely that physiological molecules involved in transmembrane signaling would use similar strategies. Furthermore, because in all cases the toxins are complicated molecules, it is unlikely that simple protein molecules will be translated unless they employ some preexisting cellular translocation mechanism.

III. PROBLEMS IN DEMONSTRATING TRANSLOCATION OF PROTEINS FROM THE EXTERIOR TO THE CYTOSOL AND TO THE NUCLEUS

In many studies on the translocation of external proteins from the exterior to the nucleus, either cell fractionation or immunofluorescence has been used. Both methods involve serious problems of interpretation, and appropriate controls are required.

In the cell fractionation approach it is not always easy to control for the possibility that the association of a protein with the nucleus could occur after the cells were disrupted or dissolved. In many cases the proteins in question contain positively charged stretches that have affinity for nuclei.

Another problem with cell fractionation as a method to assess the localization in the cell is that in some cases the receptor is anchored to cytoskeletal components that are not solubilized upon mechanical disruption of the cells or by treating them with nonionic detergents (31). Upon fractionation they may therefore appear in the same fraction as the nuclei.

A similar difficulty may arise in immunofluorescence experiments. Here the cells are often first fixed with paraformaldehyde and then permeabilized with detergents to make the intracellular proteins accessible to antibodies. A problem in these experiments is that the...
fixation does not immobilize all proteins in the cell (135). In the case of small proteins, less than half of the molecules may be immobilized. Because the fixation may damage intracellular diffusion barriers, such as the membranes of intracellular compartments, proteins that were located inside membrane-bonded vesicular structures in the living cell may diffuse into the cytosol and to the nucleus during the fixation period. Further diffusion could occur during the subsequent incubation with antibodies. As discussed in section V, some protein hormones and growth factors are, after endocytosis, transported to juxtanuclear vesicular compartments and may therefore be in an advantageous position for diffusion into the nucleus once the permeability barriers are broken down.

To test if an externally added protein is translocated to the cytosol is even more difficult than to measure transport to the nucleus. Cell fractionation experiments are here particularly difficult as both mechanical disruption and detergent lysis may liberate material from intracellular vesicular compartments such that it will appear in the cytosol fraction.

A particular problem exists in the possibility that the growth factor or a splicing variant of it may be expressed in the cytosol as a response to activation of the receptors for the same growth factor. This appears to be the case with basic fibroblast growth factor (bFGF) where the growth factor is expressed as a result of fibroblast growth factor (FGF) receptor stimulation (158). The appearance in the cytosol of the growth factor or of immunologically cross-reacting material after treatment of the cells with the same growth factor can easily be interpreted as translocation to the cytosol of the externally added protein.

Clearly, additional tests are required to demonstrate that an externally added protein is indeed transported to the cytosol and to the nucleus in vivo.

IV. NEW APPROACHES TO ASSESS THE PRESENCE IN THE CYTOSOL AND IN THE NUCLEUS OF EXTERNALLY ADDED PROTEINS

To test in a more rigorous way if externally added proteins are able to penetrate to the cytosol and nucleus, it is useful to study if enzymes that are only found in the cytosol and in the nucleus are able to modify the proteins in vivo. Two such enzymes, farnesyl transferase (231) and protein kinase C (PKC) (96, 97), have been employed. Farnesyl transferase consists of two polypeptides that are both found only in the cytosol (28, 185) and in the nucleus (121, 191). In accordance with this, both subunits are synthesized without signal sequences. The enzyme recognizes a COOH-terminal signal, a CAAX box, consisting of four amino acids. Those are cysteine followed by two aliphatic amino acids and ending with one out of several possible amino acids. The CAAX box found in K-Ras, viz. Cys-Val-Ile-Met (231), is an example. When a protein carrying this signal is added to cells in the presence of radioactive mevalonate, a precursor of the farnesyl group, the protein will only be labeled if it reaches the cytosol or the nucleoplasm in the living cell (Fig. 1).

PKC is also found only inside cells. There are many isoforms of this enzyme, but they are all found in the cytosol and in the nucleus (74, 136). A Golgi-associated form (PKC$\mu$) is found at the cytoplasmic side of the membrane as it interacts with cytoplasmic proteins (164). Therefore, if an externally added protein can be demonstrated to be phosphorylated by PKC, it follows that the protein must have reached the cytosol or the nucleoplasm in vivo (96, 97).

There are certainly many other intracellular enzymes that could be used for the same purpose. The requirement is that a similar enzymatic activity is not present at the cell surface or in intracellular vesicular or tubular structures that the protein could reach after endocytosis. It is also advantageous that the enzyme recognizes a simple amino acid sequence that can be engineered into proteins as required.

Targeting of cytosolic proteins to the peroxisomes occurs from the cytosol and requires that the protein carries a peroxisome targeting signal that may be the tripeptide Ser-Lys-Leu (196). By engineering a peroxisome targeting signal onto an external protein that is translocated into the cytosol, it is likely that it will end up in the peroxisomes where it can be visualized if the protein is fluorescently labeled. Colocalization with a peroxisomal marker protein, such as catalase, indicates its presence in the peroxisomes (193).

A fourth approach to study translocation of externally added proteins to the cytosol is to permeabilize
selectively the plasma membrane with streptolysin O. This bacterial toxin acts by forming pores in cellular membranes that are large enough to allow soluble proteins in the cytosol to diffuse out of the cell (17). It is possible to manipulate the system in such a way that pores are only formed in the plasma membrane and not in membranes of intracellular vesicles (170). The toxin acts by binding to cholesterol in the membrane, and this binding occurs even at 0°C. However, at the low temperature, the toxin is unable to form pores in the membrane. The cells are therefore extensively washed to remove unbound toxin and then exposed briefly to medium at 37°C to allow pores to be formed. If the protein leaks out from the cells under these conditions, it is likely that it is present in the cytosol (96).

V. EXTERNAL PROTEINS THAT HAVE BEEN REPORTED TO ENTER THE CYTOSOL AND NUCLEUS

A. aFGF

aFGF is synthesized without a signal peptide, and it is not known in detail how it is released from the cells. A series of papers from the laboratory of Maciag and co-workers (80) has provided information about the mechanism. In the first place, inhibitors of transport through the Golgi apparatus, such as brefeldin A, do not inhibit the release, indicating that a Golgi-dependent route is not involved. Furthermore, the release is induced by heat shock such as incubating the cells at 42°C (80).

The released growth factor is not biologically active as such, but it can be activated by treatment with high concentrations of ammonium chloride. It is released as a disulfide-linked dimer. When the cysteine residues in the growth factor were mutated, the growth factor was not released even at increased temperature (81). Further studies revealed that the critical residue was Cys-30 (210).

Together with the growth factor is released the cytoplasmic domain of synaptotagmin-1, a protein involved in membrane fusion (211). When β-galactosidase was fused to the growth factor and expressed in the cells, the whole fusion protein was exported (106). In cells transfected with a deletion mutant of synaptotagmin, neither the growth factor as such nor the fusion protein was exported. Also a member of the S100 family of calcium binding proteins, S100A13, was found to be released together with the growth factor (27). Overexpression of S100A13 allowed export even of the cysteine-free growth factor that is normally not exported (105).

Interleukin-1 (IL-1) is also synthesized without a signal sequence. When IL-1α was expressed in cells, it inhibited the export of aFGF, suggesting that they, at least partly, use the same export mechanism (212). Synaptotagmin is not required for the export of IL-1α, because the export was not inhibited by the deletion mutant that prevented export of aFGF.

aFGF is synthesized as a molecule with an extended free NH₂-terminal end. The first 20 amino acids are cleaved off in part of the molecules, but this does not appear to affect the biological activity. For this reason the shorter form is used in most experiments. The first indication that aFGF may have a function in the nucleus was obtained by Imamura et al. (77). They observed that a polybasic region close to the NH₂ terminus of the protein was required for mitogenic activity of the growth factor, but not for the ability of the growth factor to stimulate tyrosine phosphorylation and induce transcription of c-fos. When the polybasic region was replaced by the nuclear localization sequence from yeast histone 2B, mitogenic activity was restored.

Subsequent studies from the same group demonstrated that point mutations in the same region did not abolish the mitogenic effect of the growth factor and that the deletion mutant was less stable than the unmodified growth factor (59). Therefore, the NH₂-terminal nuclear localization sequence as such was not required for mitogenic activity.

Imamura et al. (79) found that the growth factor lacking a nuclear localization sequence in the NH₂-terminal region was unable to enter the nuclear fraction, whereas aFGF containing this sequence was present in the nuclear fraction obtained by cell fractionation. This was also the case when it was replaced by the nuclear localization sequence from yeast histone. However, they did not provide direct evidence that growth factor purifying together with the nucleus was really inside the nucleus in the living cell.

Later the same laboratory (78) provided immunofluorescence evidence that the growth factor really accumulated in the nuclei of BALB/3T3 cells and human umbilical vein endothelial cells, but only during the G1 phase of the cell cycle. Surprisingly, Komi et al. (101) found that a 26-amino acid peptide from the NH₂ terminus of the growth factor, including the nuclear localization sequence, was able to induce mitogenesis in cells when added in concentrations 1,000 times higher than that required for the growth factor. The mechanism behind this stimulation and if it is related to the mechanism of action of the growth factor is not known.

Sano et al. (184) found that aFGF accumulated in the nuclei in part of the cells in inflammatory arthritic joints. Later, Cao et al. (26) found that when aFGF was overexpressed in cells it was to a large extent found in the nuclei. On the other hand, they were not able to demonstrate transport of externally added growth factor to the nuclei as measured by immunofluorescence.
Prudovsky et al. (168) found that FGF receptor (FGFR)-1 moved from the cell periphery to a juxtanuclear location upon treatment of the cells with aFGF. They also found that only the receptor \( \alpha_1 \)-isoform containing three Ig-like loops, but not the receptor \( \beta_1 \)-isoform (containing only two Ig-like loops), migrated to the juxtanuclear location (169). Furthermore, the FGFR-1\( \alpha \) type of receptor was much more efficient than the \( \beta \)-type in transferring aFGF to the nuclear fraction as obtained after biochemical fractionation of the cells.

Burgess et al. (25) found that aFGF where residue 132 had been mutated to Glu (K132E) was very inefficient in inducing mitosis, although it bound to the specific FGF receptors, apparently in the same way as the wild-type growth factor. Furthermore, it induced tyrosine kinase activity of the receptor and transcription of immediate early genes like the wild-type growth factor. One difference was that the mutant growth factor bound less firmly to heparin than the wild type. Transfection of NIH 3T3 cells with the wild-type growth factor induced a transformed phenotype, but this was not the case with the mutant (24). When cells were incubated with wild-type growth factor, it was proteolytically processed to a somewhat shorter protein, but this was not the case with the mutant (66). This processing was proposed to be required for mitogenicity.

Wiedlocha et al. (233) developed a system to introduce aFGF into cells lacking receptors for the growth factor. aFGF was fused to the A-fragment of diphtheria toxin, and the toxin was reconstituted with B-fragment. This construct was able to inject the fusion protein into cells that contain toxin receptors. With the use of cells that were mutated to be resistant to the intracellular action of the toxin, it could be demonstrated that the fusion protein was translocated into the cytosol and that it subsequently moved to the nucleus. When this construct was given to cells lacking FGF receptors, DNA synthesis was induced (230). Despite this, there was no proliferation of the cells.

In further experiments it was demonstrated that for cell proliferation to occur it was also necessary that the cells expressed FGF receptors and that these were activated by the growth factor (232). It was concluded that both signals from the activated receptors and from the intracellular growth factor are necessary to induce cell proliferation.

To test if the growth factor as such, i.e., without the assistance of diphtheria toxin, is able to translocate to the cytosol and to the nucleus, two approaches were used. In one approach a farnesylation signal, a CXXC box, was added onto the COOH terminus of the growth factor (231). Because the farnesyl transferase is located intracellularly, farnesylation of the externally added growth factor indicates that the growth factor has reached the cytosol or the nucleus. It was found that the growth factor was farnesylated under these conditions. Immunoprecipitation demonstrated that the labeled protein was indeed aFGF. Binding to the specific FGF receptors was required for translocation to occur. The much more abundant binding to surface heparans did not result in translocation as measured by this procedure.

In an other approach, advantage was taken of the observation that aFGF is readily phosphorylated by PKC (95, 97). Mutation of the main site phosphorylated in vitro resulted in inability of the growth factor to be phosphorylated in vivo when given to cells. This indicates that there is a single PKC site that is phosphorylated in vivo. Because PKC is an intracellular enzyme found only in the cytosol and in the nucleus, phosphorylation of externally added growth factor can be taken as evidence that the growth factor was indeed translocated to the cytosol or nucleus.

The translocation was inhibited by genistein (146) and by wortmannin and LY294002 (94), indicating that tyrosine kinase and phosphatidylinositol 3-kinase activity are required for translocation. Mutation analysis of the FGF receptor 4 demonstrated that the kinase domain of the receptor is not necessary for translocation of the growth factor to occur. On the other hand, a short sequence in the COOH terminus of the receptor was found to be indispensable for translocation (96).

The mutant aFGF K132E referred to above (25), which binds to and activates FGF receptors in an apparently normal way, was found to be able to enter the cytosol and the nucleus (97). Furthermore, when it was introduced into cells as a fusion protein with diphtheria toxin (Fig. 2), it entered the nucleus like the wild type, but it did not induce DNA synthesis. This indicates that the mutant growth factor was unable to interact properly with an intracellular target.

Experiments were therefore conducted to search for proteins binding to the wild-type growth factor, but not to the K132E mutant. Three proteins fulfilling this criterion were indeed found. FIBP is a novel nuclear protein with unknown function (99, 100). Casein kinase 2 (CK2) is found in the cytosol and in the nucleus and consists of two subunits, both of which bind to aFGF and to bFGF. The strongest binding was seen with the enzymatically active \( \alpha \)-subunit (193). Furthermore, by comparing a number of aFGF mutants with varying mitogenic activity (95), good correlation was found between mitogenicity and binding to CK2\( \alpha \) (193). P34 is a protein associated with the cytoplasmic side of the endoplasmic reticulum (152), which also binds selectively the mitogenic growth factor (194).

Translocation of aFGF to the cytosol is inhibited by baflomycin A\(_1\) (128), which inhibits the vesicular-type proton pumps. This indicates that the translocation
occurs from an acidic vesicular compartment. However, not the acidic pH, but rather the transmembrane potential of the vesicles, is required for the translocation to occur (128). Citores et al. (31) found that in cells transfected with FGFR-4, aFGF is taken up by endocytosis that only partly is associated with coated pits. It is subsequently transported to a juxtanuclear location that was found to be identical to the recycling endosome compartment (Fig. 3). The receptor continued to signal at this location as determined by its high phosphotyrosine content.

Further studies by Citores et al. (30) demonstrated that the kinase activity of the receptor was required for endocytic uptake by the coated pit pathway, but not for uptake by an alternative pathway. The kinase activity in full-length receptor was required for efficient transport to the recycling endosomes. However, when the whole kinase domain was deleted, the growth factor was still transported to the recycling endosomes, indicating that there is a signal in the kinase domain that prevents the receptor from entering the recycling endosomes and that this signal is overruled by the tyrosine kinase activity of the receptor.

For translocation of proteins across membranes, it is usually required that the protein unfolds to a certain extent. This is also the case when aFGF is translocated into cells as a fusion protein with diphtheria toxin (230, 233). On the other hand, when aFGF as such was translocated into cells, extensive unfolding is not required (227).

**FIG. 2.** aFGF but not aFGF-K132E stimulates DNA synthesis in cells lacking FGF receptors when it is translocated into the cells with diphtheria toxin as a vector. aFGF and aFGF-K132E were fused to the NH2 terminus of diphtheria toxin A-fragment (DT-A) and reconstituted with diphtheria toxin B-fragment (DT-B) to obtain a translocation competent molecule. The constructs were given to cells lacking receptors for FGF but containing receptors for the toxin. (The cells had previously been mutated to become resistant to the intracellular action of diphtheria toxin.)

**FIG. 3.** Fluorescently labeled aFGF binds to the surface of cells transfected with FGF receptor 4. After incubation at 37°C, it is taken up by endocytosis and finally accumulates in a juxtanuclear compartment identified as the recycling endosome compartment.
B. bFGF

Very little work has been done on the endocytic uptake and intracellular transport of bFGF. Gleižės et al. (62) studied the uptake in BHK cells. The experiments were carried out in the absence of heparin, and therefore, the authors were unable to distinguish between growth factor bound to specific receptors and growth factor bound to surface heparans, which was probably the most abundant binding (175). The authors found that the growth factor was taken up from caveolae and that it was seen in early endosomes, multivesicular bodies, and lysosomes. Marchese et al. (130) studied uptake of the related keratinocyte growth factor under conditions where the binding was carried out in the presence of 0.3 M NaCl to inhibit binding to surface heparans. Keratinocyte growth factor binds to a variant of FGFR-2. They found that the growth factor was taken up from coated pits and transported to a juxtanuclear location.

Like aFGF, bFGF is synthesized without a signal sequence and is transported out of the cells by a pathway not involving the endoplasmic reticulum/GoGli route. The 27-kDa heat shock protein facilitates the export (163). Probencid (68) and ouabain (38, 57) inhibit the export, suggesting that multidrug resistance associated protein and the Na\(^+\)-K\(^+\)-ATPase are involved in the process.

bFGF is synthesized as several different molecular entities due to several CUG initiation sites upstream of the canonical AUG. The extended variants of the growth factor contain in the NH\(_2\)-terminal extensions several GR repeats that act as nuclear targeting sequences (43). Therefore, the extended forms of the growth factor are mainly found in the nucleus and are not exported out of the cells. The nuclear localization is mainly found in sparse, rapidly dividing cells, whereas in dense or senescent cells the growth factor is mainly cytosolic (238). The 18-kDa, AUG-initiated, form is found mainly in the cytosol, although some protein may also be present in the nucleus. In contrast to this, the homeoprotein engrailed contains an extension that is of importance both for the intracellular localization and for transport out of the cell (127).

When a 24-kDa extended form of the growth factor was added externally to cells, it appeared to have the same action as the 18-kDa form (67). This could be due to intracellular processing of the growth factor (141). It appears that intracellular bFGF has a different role than externally added bFGF. Thus, in neural crest-derived Schwann cells, antibodies to bFGF inhibited pigmentation caused by externally added bFGF, but it had no effect on 12-O-tetradecanoylphorbol 13-acetate-induced melanogenesis, which is dependent on intracellular bFGF (188).

Several authors have reported transport of bFGF to the nucleus after it had been added to cells. Bouche et al. (20) reported that the growth factor accumulated in the nucleoli during the G\(_1\) stage and that this correlated with the stimulation of ribosomal gene transcription in bovine endothelial cells. Externally added bFGF was found to be present in the nuclear fraction upon cell fragmentation (13). Furthermore, bFGF was reported to stimulate transcription in cell-free systems (20, 149).

When bFGF is expressed in cells, the high-molecular-mass forms are transported to the nucleus, whereas the 18-kDa form, which lacks a nuclear localization signal, is not (22). The high-molecular-mass forms contain several GR repeats (162) that are methylated and may be responsible for the nuclear localization. Transport of the endogenous growth factor to the nucleus appears to be necessary for stimulation of growth in serum-deprived cells (9, 88). The distribution of the growth factor between nucleus and cytosol was found to vary during early development (174).

By radiation fragmentation analysis it was found that bFGF is present in cells in a larger complex (156). It was later found that bFGF interacts with the \(\beta\)-subunit of CK2 and stimulates the kinase (19). The authors did not find any binding to the enzymatically active \(\alpha\)-subunit. Furthermore, they did not find any binding of aFGF to CK2. Mutation of a single amino acid in bFGF (S117A) abolished the ability of the growth factor to bind to CK2 and also abolished the mitogenic activity of the growth factor. The mutation did not abolish the ability of the growth factor to bind to and activate FGFR (11).

Altogether, there is good evidence that bFGF has an intracellular mode of action in addition to its action on the extracellular part of FGFR.

C. HIV-Tat

Tat is a protein encoded by the human immunodeficiency virus that binds to the 5′-long terminal repeats and induces strongly the synthesis of viral RNA and protein. Green and Loewenstein (63) and Frankel and Pabo (58) found independently that HIV-Tat was able to stimulate HIV-LTR-driven RNA synthesis in intact cells, particularly when chloroquine was present. Although the authors did not demonstrate directly that Tat was translocated into the cytosol and nucleus, this appears as very likely. The concentration of Tat required in the medium to obtain maximal transactivating effect was in the micromolar range. Scrape loading of the cells reduced the required concentration by a factor of 20–30. Mann and Frankel (129) found that Tat is taken up by endocytosis and that cells contain \(>10^7\) binding sites/cell. Binding, uptake, as well as the transactivation was prevented in the presence of heparin. They were unable to identify a specific receptor.

The question whether or not Tat is able to transport other proteins into cells has been approached by
several authors. Fawell et al. (55) linked Tat chemically to β-galactosidase and reported that when added to cells the whole cell was stained blue upon development with chromogenic substance. However, the resolution in these experiments was far too low to decide whether the conjugate was in the cytosol and nucleus rather than contained in intracellular vesicles. The same holds true for experiments where Schwarze et al. (187) fused TAT-translocating peptide to β-galactosidase by recombinant technology and tested uptake in cells. The fact that the cells took up the conjugate, but not β-galactosidase as such, reflects the ability of Tat to bind to surface heparans and other cell surface molecules. No evidence for penetration of the fusion protein into the cytosol or nucleus was presented.

Bonifaci et al. (18) fused dihydrofolate reductase to Tat and studied the ability of the construct to transactivate the HIV-LTR promoter of a plasmid transfected into the cells. They reported that the fusion protein transactivated LTR-CAT equally well as Tat alone. They did not exclude the, perhaps unlikely, possibility that some Tat could have been cleaved off from the fusion protein and that it entered the cytosol and nucleus without the passenger protein. Furthermore, they reported that the construct was present in the nuclear fraction when added to cells alone, but in the cytoplasmic fraction (comprising endosomes as well as cytosol) when methotrexate was present as well. Methotrexate binds to dihydrofolate reductase and prevents its unfolding (46). Unfolding is required for translocation of proteins across the membrane of the endoplasmic reticulum and of the mitochondria, and it is required for translocation of toxins across the membrane of endosomes. The data suggest that also the translocation of the fusion protein of Tat and dihydrofolate reductase requires unfolding for translocation from the exterior to the nucleus. It should, however, be kept in mind that the presence of a protein in the nuclear fraction of disrupted cells does not necessarily mean that the protein is translocated into the nucleus in vivo.

Several authors have attempted to use a presumed transducing amino acid stretch from Tat to transport passenger proteins into cells. Nagahara et al. (147) reported that an 11-amino acid sequence of Tat translocated p27kip1 into cells. This protein is a signaling protein inhibiting Cdk and arresting cells in G1. They found that the construct had this ability when added to cells, particularly if the protein had been treated with urea immediately before. They did not provide direct evidence that the protein had really been translocated into the cells, but controls with fusion protein with inactive p27kip1 suggested that this was the case.

A basic domain of Tat has been reported to enter the nucleus when added externally to cells (218). The entry was found to occur even at 4°C. Experiments to exclude that the entry of endocytosed peptide into the nucleus occurred after fixation and permeabilization are highly desirable.

Tyagi et al. (213) reported that a fusion protein of glutathione-S-transferase and Tat entered cells and induced transactivation. However, it is difficult to evaluate these data because there was a thrombin cleavage site between the two proteins and the experiment was carried out in 10% serum. It is therefore possible that the fusion protein was cleaved and that only the Tat moiety entered the nuclei.

Tat produced in HIV-infected cells or in cells transfected with the Tat gene is released from the cells and is able to stimulate growth in AIDS-KS cells at concentrations <1 ng/ml (51). On the other hand, transport to the nucleus and transactivation required a concentration of 100 ng/ml or more.

1. Tat-binding sites

The fact that Tat has angiogenic properties (4) raised the suspicion that it might bind to the receptor for an angiogenic factor. It was in fact found that Tat binds with high affinity (in the picomolar range) to one of the two receptors for vascular endothelial growth factor, VEGFR-2 or Flk-1/KDR (6, 140, 142). Binding induced the tyrosine kinase activity of the receptor. A basic peptide (amino acids 46–60) derived from Tat was also able to bind and activate the receptor, whereas another peptide (amino acids 65–80) comprising the RGD sequence was not.

At micromolar concentrations, Tat binds to certain integrins (14, 219). The basic domain of Tat was more important than the RGD domain for this binding.

A 90-kDa protein was also found to bind Tat, but this protein has so far not been identified (222). Also this protein bound to the basic peptide, but not to the RGD-containing peptide.

It has also been reported that Tat binds to the low-density lipoprotein receptor-related protein in neuronal cells (115).

Finally, Tat binds to heparin and cell surface heparan sulfate (5, 213). This is a high-capacity binding on most cells, and the affinity is much lower than the binding to the VEGF receptor. Still it is much stronger than that of cytochrome c, another basic protein.

Altogether, it is clear that Tat undergoes many interactions with cell surface molecules and induces rapidly activation of intracellular regulatory proteins such as phosphatidylinositol 3-kinase (137). Therefore, it is difficult to draw conclusions with respect to entry of the protein into the cytosol and nucleus on the basis of functional data.
D. Interferon-γ

Interferon-γ is a potent cytokine involved in regulation of many biological processes such as host defense, inflammation, and autoimmunity. The mature protein consists of 143 amino acids after cleavage of the signal sequence (54). Two interferon-γ molecules noncovalently homodimerize in an antiparallel manner (45). The interferon-γ receptor consists of a ligand-binding α-chain (3) and a β-chain required for signal transduction (72, 131, 198). The intracellular part of the interferon-γ receptor is associated with the tyrosine kinases janus kinase (JAK) 1 and JAK2 (145, 221), which upon activation of the receptor phosphorylate and activate signal transducers and activators of transcription (STAT) 1. Phosphorylated STAT1 forms homodimers (189), which are capable of activating transcription (39). However, also the interferon-γ molecule itself has been reported to enter the cytosol and nucleus to play a direct role in signaling.

Using electron microscopy with an immunogold technique, MacDonald et al. (122) reported rapid (within 1–2 min) translocation to the nucleus of prebound, extracellular murine interferon-γ in mouse L929 cells. Immune electron microscopy was also used to visualize interferon-γ in the nucleus in Hep2 cells and in mouse liver (104, 179). Similar results were obtained by cell autoradiography and by cell fractionation after incubation with 125I-interferon-γ (10).

Extracellularly added interferon-γ shows strict species specificity between human and murine cells (56, 172). This fact has been exploited in experiments suggesting that interferon-γ can trigger responses from a location in the cytosol in addition to its ability to bind to the extracellular part of interferon-γ receptors. Fidler et al. (56) used liposome encapsulation to deliver interferon-γ intracellularly. They studied interferon-γ-stimulated tumoricidal properties of purified human blood monocytes and mouse peritoneal exudate macrophages. In a first set of experiments to confirm the species specificity, they found that extracellular human interferon-γ (together with muramyl dipeptide) stimulated the tumoricidal activity of human monocytes, but not of mouse macrophages, and vice versa, extracellular mouse interferon-γ stimulated the mouse cells, but not the human ones. In contrast, when liposome-encapsulated interferon-γ was used, the effects were no longer species specific. Furthermore, pronase treatment of the cells to remove cell surface interferon-γ receptors rendered the cells insensitive to unencapsulated interferon-γ, while liposome-encapsulated interferon-γ still stimulated tumoricidal activity.

Smith et al. (197) found that human interferon-γ stimulated expression of a major histocompatibility complex (MHC) class II molecule (Ia) when microinjected into murine macrophages, but not when added extracellularly. Sanseau et al. (180) took another approach by expressing in mouse L cell lines wild-type or a truncated version of human interferon-γ lacking the signal sequence for secretion. Mouse cells expressing intracellular, nonsecreted human interferon-γ were protected against viral infection (varicella-zoster and Mengo virus), while transfectants secreting human interferon-γ were susceptible to infection. Protection by intracellular human interferon-γ was slightly less efficient than protection by extracellular murine interferon-γ. Also, expression of MHC class II antigens was induced by intracellular, but not by secreted, human interferon-γ in these mouse cells.

Similar results were obtained by Lewis et al. (109), who first expressed murine interferon-γ lacking its signal sequence in human A-549 cells, thereby obtaining cells partially protected against viral infection. Next, these authors changed to using a similar approach in a murine cell line, designated Ltk-aprt-cells, which is resistant to the antiviral effects of both interferon-γ and interferon-β, but becomes protected against viral infection by the simultaneous stimulation by these factors. Expression of nonsecreted human interferon-γ was shown to cooperate with extracellularly added murine interferon-β. In control experiments they showed that this effect was not due to leakage to the medium of human interferon-γ, since extracellular human interferon-γ had no effect on the cells. Also, the observed effect was not due to expression of murine interferon-γ. The advantage of the rather complicated cell-ligand system used in these experiments is that it rules out the possibility that secretion of endogenous type I interferons (α or β) rendered the cells resistant. Together, this work suggests that, in addition to the species-specific binding to and activation of cell surface receptors, interferon-γ may also exert species nonspecific effects in the cytoplasm.

This notion was supported by the finding of a non-species specific binding site for interferon-γ on the cytoplasmic part of interferon-γ receptor α-subunit (64, 208, 209). A sequence in the NH2 terminus of interferon-γ bound to the extracellular part of the receptor, while a COOH-terminal stretch of interferon-γ was able to bind to the intracellular part of the receptor. The intracellular binding site for interferon-γ on the receptor was reported to be required both for internalization of the ligand and for biological responsiveness (53). Furthermore, Szente et al. (209) reported that a peptide derived from the COOH-terminal part of interferon-γ could induce viral resistance and MHC class II upregulation in a murine macrophage cell line in a rather species nonspecific manner.

The COOH termini of both murine and human interferon-γ contain a basic amino acid stretch similar to the nuclear localization signal (NLS) of simian virus 40 T-antigen. This sequence was shown to be a functional NLS
in a standard nuclear import assay in digitonin-permeabilized cells (207). Also, this sequence appears to be crucial for the biologic activity of the molecule (8, 120, 195, 229).

Subramaniam et al. (206) studied nuclear translocation of STAT1 in response to interferon-γ. Preincubation of interferon-γ with polyclonal antibodies against its COOH-terminal, NLS-containing part blocked STAT1 nuclear translocation. The same effect was obtained by deleting the NLS of interferon-γ. Microinjection of the antibodies into mouse L929 cells also inhibited STAT1 nuclear translocation in response to interferon-γ, but not in response to interferon-α, suggesting a direct role of cytoplasmic interferon-γ in the nuclear translocation of STAT1. In support of this hypothesis, interferon-γ and STAT1 were coimmunoprecipitated with NPI-1 (importin-α homolog) from cell lysates after the cells had been treated with interferon-γ at 37°C. Less interferon-γ was coimmunoprecipitated from cells treated at 4°C or treated with interferon-γ with deleted NLS.

Altogether, there is considerable evidence for a role of interferon-γ in the cytosol and nucleus.

E. Other External Proteins That May Enter the Cytosol and Nucleus

1. Vascular endothelial growth factor

Li and Keller (111) demonstrated that when a monolayer of bovine adrenal cortex cells was wounded by a scratch, the cells close to the wound started to take up vascular endothelial growth factor (VEGF) in their nuclei. Cells located more distantly from the wound took up the growth factor by endocytosis, but in this case without nuclear localization. The nuclear uptake lasted for a few hours and resulted in induction of a number of proteins that were not expressed in the more distantly located cells. Binding of the growth factor to the specific VEGF receptors was required for nuclear accumulation. Several control experiments were conducted to exclude the possibility that the nuclear localization was due to injury of the cell membrane in the cells close to the scratch, which could conceivably have allowed entry of the growth factor through membrane wounds with subsequent accumulation in the nucleus. Interestingly, as the cultures grew older, they lost the ability to respond to injury with transport of VEGF to the nuclei.

2. Schwannoma-derived growth factor

Kimura (90) studied the interaction of oligonucleotides with Schwannoma-derived growth factor, a member of the EGF growth factor family (and the rat equivalent of amphiregulin). They found that it interacts with AT-rich sequences. Differently from EGF, the Schwanna-derived growth factor contains a NLS. When this was mutated, the growth factor lost its mitogenic effect, although it retained its ability to bind to and activate EGF receptors. This suggests that the growth factor has an intracellular site of action in addition to its activation of the receptor. Further evidence for this is that Schwannoma-derived growth factor was able to stimulate DNA synthesis in cells where the kinase domain of the EGF receptor was inactivated by mutation, whereas EGF, which does not contain a NLS, lacked this ability.

3. Heregulin

Heregulin is another growth factor of the EGF family. This protein also contains a NLS. Li et al. (112) found that the growth factor was accumulated in the nuclei 30 min after being added to the cells. This was demonstrated by immunofluorescence and by electron microscopy with 125I-labeled growth factor. It would have been desirable to demonstrate the nuclear labeling by additional methods to exclude the possibility of artifacts.

4. Parathyroid hormone-related protein

Parathyroid hormone-related protein (PTHrP) has been found in the nuclei of several authors. This protein has several splice variants in the range of 139–173 amino acids. The NH2 terminus is identical to that of parathyroid hormone, and therefore, it binds to the parathyroid hormone receptor, a G protein-linked heptahelical transmembrane protein. PTHrP is responsible for the common endocrine paraneoplastic syndrome humoral hypercalcaemia (23). When the protein was overexpressed in cells, it was detected both at the cell surface, in the cytosol, and in the nucleus (2, 132). The protein contains a nuclear targeting sequence, and when this was mutated, both surface binding of the protein and accumulation in the nucleus were eliminated. The authors found that when a small peptide containing the nuclear targeting sequence was added to cells, it was accumulated in the nucleus both in cells containing PTHrP receptors and in cells lacking them.

The import of PTHrP into the nuclei is apparently due to a direct interaction with importin-β, whereas importin-α does not seem to be involved (103). PTHrP was found to bind tightly to RNA (1).

Prepro-PTHrP expressed in cells was found to be degraded by a process involving ubiquitinylation and degradation by proteasomes (134). This indicates that the protein can be translocated retrograde from the endoplasmic reticulum to the cytosol. If some of it is not degraded, it could accumulate in the nucleus. There is, however, no evidence that externally added PTHrP is translocated by this route as has been demonstrated in the case of ricin (226). Altogether, there is so far insufficient data supporting the view that externally added PTHrP is translocated to the nucleus in vivo.
5. Prolactin

Prolactin was reported to be present in the nucleus of T lymphocytes that had been treated with IL-2 (35). The data were obtained with immunofluorescence of fixed cells. Antiprolactin prevented growth of IL-2-stimulated cells, but when prolactin containing a NLS and no signal sequence was expressed in the cells, they were growing in the presence of IL-2 (34). Expression of prolactin only lacking the signal sequence did not stimulate growth. This suggests that prolactin has an intracellular, probably nuclear, action in addition to its interaction with the cell surface receptors. Later work from the same group (15) reported that the translocation of prolactin to the nucleus was inhibited by rapamycin, wortmannin, and LY294002, indicating that phosphatidylinositol 3-kinase is involved.

Perrot-Applanat et al. (160) found no evidence for transport of prolactin or its receptor to the nucleus, although both were extensively endocytosed and accumulated in a juxtanuclear position.

Rycyzyn et al. (178) reported that cyclophilin B binds to prolactin and is endocytosed together with the prolactin–prolactin receptor complex. The complex is then transported retrograde in the cell to the endoplasmic reticulum where prolactin and cyclophilin are translocated to the cytosol and subsequently transported to the nucleus. Removal of a nuclear targeting signal from cyclophilin abolished its ability to stimulate DNA synthesis.

6. Growth hormone

Growth hormone and its receptor have also been found to be associated with the nucleus (116, 117). The evidence was based on cell fractionation experiments and immunofluorescence. In addition, some experiments with $^{125}$I-labeled growth hormone and electron microscopy were carried out. Neither of these methods is sufficiently sensitive to exclude the possibility that the receptor and the growth hormone were in juxtanuclear membrane-bound compartments rather than inside the nucleus. In any event, the authors observed growth hormone-stimulated transport of the growth hormone receptor to the nuclear region, consistent with, but not proving, that the ligand induced translocation of the receptor to the nucleus.

7. IL-1α

Grenfell et al. (65) found that IL-1α bound strongly to isolated nuclei. Cell fractionation studies demonstrated that IL-1α added to cells was endocytosed and accumulated in the nuclear fraction. Curtis et al. (37) and Weitzmann et al. (223) reported that IL-1α enters the nucleus together with its receptor. In both cases it was not excluded that the lymphokine was bound to its receptor located in a juxtanuclear area in such a way that the interleukin was still inside a vesicular structure. Upon fractionation of the cells, the complex could be present in the nuclear pellet. Maier et al. (126) found that the precursor of IL-1α expressed in cells accumulated in the nucleus and that this was necessary to inhibit growth in some cells, whereas it acted as an oncoprotein in others (205). The ability to accumulate in the nuclei was found to reside in the NH$_2$-terminal part of the precursor that is not present in the exported protein. Also, the NH$_2$-terminal prodomain of IL-16 was found to localize the lymphokine to the nucleus (239).

8. Hepatoma growth factor

Hepatoma growth factor belongs to a new family of growth factors characterized by a so-called HATH box (91). It is synthesized without a leader sequence, but it has a bipartite NLS. Externally added growth factor was reported to be internalized and enter the nuclei. The evidence for this was immunofluorescence studies (91), and it will be interesting to see this corroborated by additional evidence.

9. Cytokines and growth factors

Several cytokines and growth factors have been found to act in an autocrine way. In typical experiments antibodies against the factor added to the medium did not inhibit the stimulatory effect, whereas the expression of antisense nucleotides did inhibit it. This was found for IL-6 (118, 177). However, this does not necessarily mean that the cytokine acts in the cytosol or nucleus. An alternative explanation is that the cytokine binds to and signals from receptors present in intracellular vesicular compartments. On the other hand, the recent finding by Yu et al. (237) that endocytosed IL-2 bound to its receptor is degraded by proteasomes indicates that the interleukin is translocated, presumably from the endoplasmic reticulum to the cytosol. If some of the retrograde translocated protein is not degraded, it could exert an intracellular action either in the cytosol or in the nucleus.

In the case of platelet-derived growth factor, an alternative B-chain-like protein has been described that lacks the signal sequence and is therefore expressed in the cytosol (42) and possibly in the nucleus.

Wetmore et al. (228) found that brain-derived growth factor is present in the nuclei of cells producing the growth factor. Possibly, this is due to translation initiation at an upstream CUG initiation site, thus initiating without an NH$_2$-terminal signal sequence.

Macrophage inhibitory factor was found to interact with an intracellular protein Jab1, which is a coactivator of AP-1 (92). Although direct evidence for translocation of this 12-kDa protein from the exterior to the cytosol is lacking, the data suggest that the cytokine has an intracellular action.
10. VP22

A protein from herpesvirus, VP22, was reported to have the ability to exit the cell where it is synthesized and enter adjacent cells where it was found in the nucleus in immunofluorescence studies (47). The protein was also reported to carry passenger proteins, such as p53, into cells and deliver them to the nucleus (16, 161). However, the protein was unable to carry diphtheria toxin A-fragment into the cytosol (52), and recent studies (119) have raised questions whether the protein is redistributed after the (incompletely) fixed cells were rehydrated. Further experiments are required to settle this question.

11. Insulin

It has long been discussed whether or not insulin has a cytosolic mechanism of action. Immunofluorescence and other indirect methods have indicated the presence of the hormone in the cytosol, and an intracellular binding protein has been identified (70). Microinjection of insulin stimulated RNA and protein synthesis in frog oocytes (138). However, more definitive evidence is required before it can be concluded that insulin is able to penetrate into cells.

Also, insulin-like growth factor has been reported to be translocated into cells and to the nucleus, and specific binding proteins are also found to enter cells (186). Also in this case, the evidence is only circumstantial and can be interpreted in alternative ways.

12. Connective tissue growth factor

Connective tissue growth factor has mitogenic activity and is also involved in cell adhesion, angiogenesis, and cell migration. It is a cysteine-rich glycoprotein with a molecular mass of 36–38 kDa. Recently, evidence was presented that when given extracellularly it is able to penetrate to the cytosol and to the nucleus (220). This was demonstrated by the ability of the cells to phosphorylate the externally added growth factor, apparently by PKC.

13. Lactoferrin and α-lactalbumin

Two unlikely proteins to enter the nucleus after having been applied to intact cells are multimeric lactoferrin and α-lactalbumin. Externally added lactoferrin was reported to bind to specific sequences in DNA and to activate transcription (71). Hakansson et al. (69) found that multimeric α-lactalbumin induces apoptosis in tumor cells, and immunofluorescence data suggested that the protein enters the nuclei. In both cases, more direct evidence is necessary before it can be established that these two proteins really possess the ability to enter the nuclei of living cells.

14. Ciliary neurotrophic factor

Ciliary neurotrophic factor lacks a signal sequence. It was found to be present in the nuclei of cells producing it (12), despite the fact that it lacks a typical NLS. Perhaps its small size allows it to enter the nucleus where it could be bound to an acceptor molecule. Its role in the nucleus is not known.

15. Angiogenin

Angiogenin is a 14-kDa protein belonging to the pancreatic RNase superfamily. It induces growth of endothelial cells. A putative NLS is required for this activity. Immunofluorescence data have indicated that this protein is able to enter cells from the exterior and that it is transported to the nucleus where it accumulates in the nucleolus (110, 143, 144). The protein was found to accumulate in the nucleolus very rapidly. Unfortunately, evidence that the nucleolar localization was not due to a redistribution during or after fixation was not provided.

A basic peptide derived from FGF-4 was used to translocate Cre recombinase into cells (84, 157).

It may be concluded that many observations suggesting that a variety of cytokines and growth factors have intracellular functions. Many of these proteins have sequence stretches rich in basic amino acids that resemble NLSs. In some cases they have even been reported to target passenger proteins to the nucleus. Removal or mutation of these sequences has in several cases changed or eliminated their biological activity or part of it (82, 83). In some cases microinjection or expression of the growth factor or cytokine in the cytosol has elicited the same effect as external addition of the protein. Although these are interesting observations compatible with intracellular actions of the proteins, many of them can also be explained in alternative ways, and more direct evidence is required before it can be established that the proteins act intracellularly.

VI. TRANSPORT OF TRANSMEMBRANE RECEPTORS TO THE NUCLEUS

Many growth factor and cytokine receptors have been reported to be present in the nucleus (37, 117, 124, 216, 235).

Several groups have reported that FGF receptors are found in the nucleus. Johnston et al. (86) found that a splicing variant lacking the transmembrane domain of FGFR-3 was found in the nuclei of mammary cancer cells. When they expressed in cells a similar construct that also lacked the signal sequence, it was efficiently transported to the nucleus.

C-erbB-4/HER-4, a receptor related to the EGF receptor, was found by immunostaining with two different
antibodies to be localized to the nucleus in some, but not all, breast cancers tested (200). Recently, it was observed
that the cytoplasmic part of this receptor is cleaved off by
\( \gamma \)-secretase and enters the nucleus (150) in a similar fash-
on as Notch (89).

It has been reported that EGF receptor is transferred
to the nuclei upon stimulation with its ligand (73) and that
it has transactivating activity (113). The presence of the
receptor in the nuclei was studied by confocal micros-
copy (113), which indicated that it was located as an outer
rim around the nuclei. It is therefore possible that the
receptor is in the nuclear envelope rather than inside the
nucleus as such. If it is inserted into the inner nuclear
membrane, the cytoplasmic tail of the receptor could
be in contact with chromatin and thus have a transactivating
effect. Further studies are necessary to elucidate this
question.

It is mechanistically more difficult to understand how
FGFRs that are inserted into the membrane become trans-
located to the nucleus upon stimulation with bFGF as
demonstrated by confocal and electron microscopy and
by cell fractionation (123, 125, 159, 201–204). After bind-
ing of FGF to its receptors, the complex is internalized by
a process that at least partly involves coated pits (199).
The import of FGFR-1 to the nucleus induced by the
18-kDa form of bFGF involves importin-\( \beta \) (173). It ap-
pears that it is the full-length receptor that is transported
to the nucleus and not only the cytosolic part as in the
case of Notch (89). In bovine adrenal medullary cells, the
translocation of the FGFR to the nucleus results in trans-
activation of angiotensin responsive elements in the
bFGF promoter (158). This is important to keep in mind
as it indicates that addition of bFGF to cells may induce
synthesis of bFGF in the cells, which could erroneously
be interpreted as evidence for transport of the added
bFGF into the cells.

Recent studies on the ERAD of proteins (75, 102, 176,
224, 234, 236) could explain how such translocation could
take place. It was found that whole transmembrane pro-
teins were translocated retrograde to the cytosol and then
degraded by the proteasomes. The proteasomes may even
participate in the translocation process (85, 176). When
proteins are overexpressed, the proteasomes may be-
come overloaded, and some protein may then accumulate
in the cells as aggresomes (87). It is possible that this
system could be modified in such a way that the protein
could be released to the cytosol in an intact form and
carry out intracellular functions as was indeed found in
the case of ricin (226).

It is particularly interesting that some growth factor
receptors accumulate at the nuclear membrane (31, 113).
In the case that the receptor is inserted in the inner
nuclear membrane, the otherwise cytoplasmic tail could
interact directly with components in the nucleus. Struc-
tures in the nucleus could assist in releasing the growth
factor from the membrane and transport it into the nu-
cleus. Although this is mainly speculation at the present
time, it indicates that the idea that growth factor recep-
tors in their intact form can be translocated to nucleus in
vivo should not be dismissed as biologically inconceiv-
able.

VII. CONCLUDING REMARKS

A large amount of evidence suggests that many pro-
tein hormones and growth factors as well as their recep-
tors may act intracellularly, preferentially in the nucleus.
In many cases the data are insufficient to conclude that
the necessary translocation across cellular membranes
has indeed taken place. However, the emerging picture is
that at least in some cases growth factors and cytokines
do translocate from the cell exterior to the cytosol and
nucleus. Particularly in the case of aFGF, bFGF, HIV-Tat,
and interferon-\( \gamma \) there is reason to believe that transloca-
tion of biologically relevant amounts does occur. In sev-
eral other cases, biological effects consistent with trans-
location were observed, but these effects could either be
explained in alternative ways or the test system was
extremely sensitive. It is known from work with toxins
that the enzymatic subunit, which is not able to efficiently
penetrate by itself, may be toxic if added at concentra-
tions thousands to a million times higher than that re-
quired for the whole toxin. Apparently, a few molecules
may sicker through under these conditions. How this
occurs is at present unknown, but it could be by retro-
grade transport to the endoplasmic reticulum with subse-
quent translocation by the misfolded protein pathway. It
is unlikely that such translocation plays important physi-
ological roles because the high extracellular concentra-
tions required of the protein in question is unlikely to be
reached in vivo. At least in the case of aFGF, translocation
is now partly characterized in that it requires the specific
receptors, it probably occurs from endocytic vesicles, it
requires a membrane potential across the vesicular mem-
brane, and it is dependent on active phosphatidylinositol
3-kinase. It remains to be tested if the other proteins are
translocated by the same or by different mechanisms.

Should it turn out that translocation of protein hor-
mones, growth factors, and cytokines to the cytosol and
nucleus is a widespread phenomenon, it could have con-
siderable consequences for our understanding of the way
signal is transferred from the exterior to the interior of
cells.

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